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Letter to the Editor

ESAT-6 and CFP-10: What Is the Diagnosis?

In a recent issue of *Infection and Immunity*, Geluk and coworkers (7) reported the observation that human T cells from both *Mycobacterium leprae*- and *Mycobacterium tuberculosis*-sensitized individuals recognize the *M. leprae* ESAT-6 orthologue. This has prompted us to comment on the potential use of these antigens as diagnostic markers, as well as to comment on the use of the misnomer "*M. tuberculosis* specific" when referring to these antigens.

The use of the designation "*M. tuberculosis* specific" has grown steadily over the last few years as the number of studies on the potential use of ESAT-6 and CFP-10 as diagnostic markers for *M. tuberculosis* infection has increased. Contrary to common belief (e.g., see references 1 to 3, 6, and 12), the secreted *M. tuberculosis* ESAT-6 and CFP-10 T-cell antigens are not *M. tuberculosis* specific (8). Despite earlier evidence to the contrary (9), orthologues of ESAT-6 and CFP-10 are

ESAT-6

Mouse T-cell epitope and Human T-cell epitope - India (aa 1-20)
Human T-cell epitope - Germany (aa 1-30)
Human T-cell epitope - Ethiopia (aa 42-75)
Mouse T-cell epitope and Human T-cell epitope - India (aa 51-70)

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M. tb     MTEQQWNFAGIEAAASAIQGNVTSIHSLLDEGKQSLTKLAAAWGGSGSEAYQGVQQKWDA
M. smeg   MTEQVWNFAGIEGGASEIHGAVSTTAGLLDEGKASLTTLASAWGGTGSEAYQAVQARWDS
          **** *******..** *:* *::  .****** ***.**:****:******.** :**:
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Human T-cell epitope - India (aa 66-90)
Human T-cell epitope - Kuwait and Denmark (aa 72-95)

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M. tb     TATELNNALQNLARTISEAGQAMASTEGNVTGMFA 95
M. smeg   TSNELNLALQNLAQTISEAGQTMAQTEAGVTGMFA 95
          *:.*** ******:*******:**.**..******
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CFP-10

Human T-cell epitope - India (aa 16-30)
Human T-cell epitope - India (aa 51-70)

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M. tb     MAEMKTDAATLAQEAGNFERISGDLKTQIDQVESTAGSLQGQWRGAAGTAAQAAVVRFQE
M. smeg   MAAMNTDAAVLAKEAANFERISGELKGVIAQVESTGSALAAQMVGQAGTAAQAALARFHE
          ** *:****.**:**.*******:**  * *****..:* .*  * ********:.**:*
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Human T-cell epitope - India (aa 71-90)

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M. tb     AANKQKQELDEISTNIRQAGVQYSRADEEQQQALSSQM 98
M. smeg   AAAKQVQELNEISANIHTSGTQYTSTDEDQAGTLASSM 98
          ** ** ***:***:**: :*.**: :**:*  :*:*.*
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FIG. 1. Alignment of the protein sequences of the ESAT-6 and CFP-10 orthologues from *M. tuberculosis* (*M. tb.*) and *M. smegmatis* (*M. smeg.*). Although studies have indicated the presence of multiple T-cell epitopes scattered throughout the ESAT-6 protein sequence (10, 11, 15), the positions of predominantly recognized epitopes are underlined (4, 10, 11, 12, 15). Asterisks indicate identical amino acid residues; colons and dots indicate conserved and semiconserved substitutions, respectively, according to their physiochemical criteria. aa, amino acid.

present in the genomes of *M. leprae* and even the distantly related, nonpathogenic, fast-growing, environmental mycobacterium *M. smegmatis* (8). This is further supported by results dating back to 1995, which showed that the genes for these proteins are also present in other pathogenic mycobacteria (*M. africanum*, *M. kansasii*, *M. marinum*, and *M. szulgai* [9, 14] and *M. bovis* [9]) as well as the slow-growing nonpathogenic mycobacterium *M. gastri* (5) and the fast-growing nonpathogenic environmental species *M. flavescens* (9).

This raises a question concerning the potential use of these antigens as diagnostic markers. We have previously suggested that the presence of orthologues of ESAT-6 in other mycobacterial species may influence the use of ESAT-6 as a diagnostic marker for *M. tuberculosis* infection (8). The results presented by Geluk and coworkers (7) that show significant cross-reactivity between the *M. tuberculosis* ESAT-6 and its orthologue from *M. leprae* support our viewpoint. The investigators came to the conclusion that this significant cross-reactivity indicates low specificity and has implications for its use as a diagnostic tool in areas where both tuberculosis and leprosy are endemic.

The similarities between the *M. tuberculosis* ESAT-6 and CFP-10 proteins and their orthologues in *M. smegmatis* are 80 and 71%, respectively (Fig. 1), whereas *M. leprae* ESAT-6 shares a much lower amino acid sequence similarity with *M. tuberculosis* ESAT-6 of around 63%. Therefore, it is likely that these proteins also share epitopes that may result in cross-reactive T-cell responses. Furthermore, given the evolutionary history of the mycobacteria (13) and the presence of ESAT-6 and CFP-10 in *M. smegmatis* and other mycobacterial species, it is plausible that these genes would be present in the genomes of most other environmental mycobacteria. The homology between ESAT-6 and CFP-10 of the many environmental mycobacterial strains phylogenetically more closely related to *M. tuberculosis* may even be higher than that between the antigens of *M. tuberculosis* and *M. smegmatis* (Fig. 1). We believe that there is an urgent need to study the extent of amino acid sequence similarity between the ESAT-6 and CFP-10 proteins of different pathogenic and nonpathogenic environmental mycobacteria, as well as the influence of secreted ESAT-6 and CFP-10 from environmental mycobacteria on the T-cell responses from *M. tuberculosis*-infected individuals. Gamma interferon production in response to ESAT-6 and CFP-10 from environmental mycobacteria by peripheral blood mononuclear cells from infected patients has, to our knowledge, not been studied. This is surprising, given the fact that numerous studies have already been done on the use of these antigens as diagnostic tools (see, for example, references 1 to 3 and 12). Results are still needed which indicate that the host cellular immune response is able to distinguish between the ESAT-6 and CFP-10 proteins secreted from either environmental mycobacteria or *M. tuberculosis*.

It is possible that the promising results obtained with ESAT-6 and CFP-10 in industrialized countries may be of less benefit to people living in developing countries where environmental mycobacteria are present in large amounts and where the real need for these tests lies.

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Nico C. Gey van Pittius*
Robin M. Warren
Paul D. van Helden
US/MRC Centre for Molecular and Cellular Biology
Department of Medical Biochemistry
Faculty of Health Sciences
University of Stellenbosch
PO Box 19063
Tygerberg 7505, South Africa

*Phone: 27-21-938-9402
Fax: 27-21-938-9476
E-mail: ngvp@sun.ac.za

Authors' Reply

We read with interest the letter of Dr. Gey van Pittius et al. in response to our recent paper (6). Both the ESAT-6 and CFP-10 antigens have over the years been analyzed extensively for their distribution in mycobacterial species (13), and their diagnostic relevance has been discussed (11). As Dr. Gey van Pittius et al. state, thus far homologous genes have been detected in a number of species outside the *Mycobacterium tuberculosis* complex. As a consequence, the expression of these antigens in other mycobacteria may in theory confound specific diagnosis of *Mycobacterium tuberculosis* and *Mycobacterium bovis* infections.

The question, however, is what consequences does this have for clinical and epidemiological practice in tuberculosis control. The presence of ESAT-6 and CFP-10 homologues in other species, for example, does not seem to confound the detection of *M. tuberculosis*-associated specific responses in the large number of studies conducted by different groups over the last 5 years (1–3, 9, 10). At present it remains unknown whether these genes are truly expressed in nonpathogenic as opposed to pathogenic mycobacteria and if the amino acid identity observed is enough to trigger a highly specific T-cell response. Particularly important for this discussion is the observation that T-cell responses to ESAT-6 and CFP-10 are apparently associated with active ongoing infection and as such have prognostic protential (5, 14). Therefore, T-cell responses to these antigens are presumably not associated with exposure to nonpathogenic strains such as *M. smegmatis* and *M. scrofulaceum*. Even in highly sensitive enzyme-linked immunospot assays that detect single ESAT-6-positive T cells, control individuals were negative (8). This conclusion is also supported by the many studies conducted in cattle where these reagent are highly specific indicators of ongoing *M. bovis* infection although cattle must be exposed daily to nonpathogenic mycobacteria from soil and natural water sources (4, 12, 14). That (rare) clinical infection with the two pathogenic strains *M. marinum* and *M. kansasii*, on the other hand, actually can trigger ESAT-6- and CFP-10-specific T-cell responses was recently convincingly demonstrated (S. M. Arend, K. E. van Meijgaarden, K. de Boer, E. Cerdá de Palou, D. van Soolingen, T. H. M. Ottenhoff, and J. T. van Dissel, submitted for publication). The same holds true for *M. leprae* as discussed above (6). While some caution may therefore be needed in the immunodiagnosis of clinical tuberculosis since infections with these three pathogens cannot be excluded by ESAT-6- and CFP-10-based tests, in practice only infections with *M. kansasii* may, though rarely, pose a differential diagnostic problem.

We agree with Dr. Gey van Pittius et al. that there is an urgent need for good diagnostic tools in the developing world. We assume that the major complicating factor for the application of reagents such as ESAT-6 and CFP-10 in the diagnosis of tuberculosis in countries of endemicity, however, is not the presence of environmental mycobacteria but the enormous reservoir of latent human tuberculosis (7).

Thus, despite the fact that antigens such as ESAT-6 and CFP-10 are not restricted to *M. tuberculosis*, they hold promise for the specific detection of *M. tuberculosis* infection.

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A. Geluk
T. H. M. Ottenhoff*
Department of Immunohematology and Blood Transfusion
Leiden University Medical Center
PO Box 9600
2300 RC Leiden, The Netherlands

S. M. Arend
Department of Infectious Diseases
Leiden University Medical Center
2300 RC Leiden, The Netherlands

P. Andersen
T. M. Doherty
Department of TB Immunology
Statens Serum Institute
Copenhagen, Denmark

*Phone: 31-71-5263800
Fax: 31-71-5216751
E-mail: t.h.m.ottenhoff@lumc.nl